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Prevalence and natural host range of Homalodisca coagulata virus-1 (HoCV-1)

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Summary

Transmission electron microscopy was used to confirm the presence of picorna-like virus particles presumed to be Homalodisca coagulata virus-1 (HoCV-1) in the midgut region of adult glassy-winged sharpshooters (GWSS). In addition, we offer a reverse transcription polymerase chain reaction (RT-PCR) assay for the detection of this virus with a sensitivity of ~95 genome equivalents. A survey employing this assay in conjunction with GWSS samples collected throughout the United States including California, Hawaii, Florida Georgia, and the Carolinas revealed a fairly widespread pattern of distribution, although potentially restricted to temperate regions, areas with elevated host densities, or to populations of a common origin. The virus was found to naturally infect adults regardless of host plant and was not specific to a particular lifestage or sex. Examination of alternate leafhopper species further demonstrated that, although infection is not ubiquitous to all sharpshooter genera, HoCV-1 is not limited to *Homalodisca vitripennis* (=*H. coagulata*).

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Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (=*H. coagulata* (Say) (Hemiptera: Cicadellidae) [17]), is a highly mobile, polyphagous pest which feeds on over 100 plant species in 31 families [2, 6]. Indigenous to the southern United States and northeastern Mexico, GWSS have recently invaded and successfully occupied the French Polynesian island of Moorea, Easter Island [13], and coastal areas of Tahiti [3] as well as the Hawaiian island Oahu [5]. Throughout these regions, this insect has emerged as a significant threat to both ornamental and agricultural operations due to its ability to vector *Xylella fastidiosa* Wells. Upon infection, the bacterium obstructs xylem flow, manifesting infection through scorch-like symptoms and ultimately plant death [19]. Strains of this phytopathogenic microbe have been associated with a number of diseases including leaf scorches of almond, coffee, elm, maple, oak, oleander, and pear as well as citrus variegated chlorosis, phony peach disease, and Pierce's disease of grapes [10, 14, 20].

While application of pyrethroid and neonicotinoid insecticides continues to be the first line of defense against the GWSS in large-scale commercial vineyards and orchards, many producers are moving away from broad-spectrum chemical control to more environmentally "benign" pest management strategies. Currently, two species of entomopathogenic fungi, *Pseudogibellula formicarum* (Mains) Samson & Evans and *Metarhizium anisopliae* (Metchnikoff), and four mymarid wasps comprise the arsenal of available self-sustaining, biological control agents against this insect vector [8, 9]. In addition, a single-stranded RNA (ssRNA) virus was recently discovered through sequencing of expressed sequence tags (ESTs) derived from GWSS adults collected from a citrus grove in Riverside, California [7]. A comprehensive molecular characterization and phylogenetic analysis of this novel virus, denoted Homalodisca coagulata virus-1 (HoCV-1), led to its tentative placement within the genus *Cripavirus* (family *Dicistroviridae*). Previous studies examining the pathological effects of members of related virus species have shown increased mortality rates, delayed development, and reduction in fecundity – factors vital to the establishment of viable, efficient biopesticides [11, 12, 18].

Because HoCV-1 was initially detected in California GWSS, it was uncertain as to whether the virus occurred in Florida populations. To address this question, a simple, rapid, cost-effective method for detection of HoCV-1 using reverse transcription polymerase chain reaction (RT-PCR) was developed. Employing this assay, we sought to establish if a correlation existed between HoCV-1 susceptibility and insect sex or lifestage. In addition, a preliminary study was undertaken to determine the occurrence of HoCV-1 in GWSS populations throughout Florida. GWSS having geographically disparate origins (Georgia, South Carolina, North Carolina, and Hawaii) were also tested. A secondary investigation was conducted to gauge whether HoCV-1 is restricted to *H. vitripennis* or, alternatively, if the virus naturally infects other sharpshooter vector species. Hypotheses which attempt to explain the incidence of the virus in heterogenic leafhopper populations are discussed.

Methods

Insect sampling

To gain insight into whether HoCV-1 infects only certain stadia, adult GWSS were collected from the University of Florida North Florida Research & Education Center (Quincy, FL) and transferred to the USDA ARS U.S. Horticultural Research Laboratory (Ft. Pierce, FL), where they were managed as a colony until all life stages could be collected. Individual growth stages were determined according to head capsule width as described by Sétamou and Jones [15]. In an attempt to avoid contamination of egg tissue with potentially infected plant material, egg masses were dissected away from the leaf prior to processing.

Adult leafhoppers were collected over a two-year period from various geographical locations throughout the United States including Florida, Georgia, South Carolina, North Carolina, California, and Hawaii. Additional sharpshooter vector species included in the study were as follows: black-winged sharpshooter [*Oncometopia nigricans* (Walker)], blue-green sharpshooter [*Graphocephala atropunctata* (Signoret)], green sharpshooter [*Draeculacephala minerva* Ball], and Johnsongrass sharpshooter [*Homalodisca insolita* (Walker)]. All samples were placed into individual microfuge tubes to prevent cross-contamination of any virus which may have been emitted via aerosols or excreta. Insect tissue was preserved in RNA*later*® (Ambion, Woodward, TX) and maintained at $-20\,^{\circ}C$ until total RNA could be extracted.

Transmission electron microscopy

Infected GWSS adults were transected into three segments (head, thorax and abdomen) and fixed with 3% glutaraldehyde in Sorenson's Na–K phosphate buffer for 1 h at room temperature. Following a single overnight and two 2 h rinses in fresh buffer, specimens were postfixed in 1% osmium tetroxide for 1 h at room temperature. Samples were rinsed in buffer and subsequently dehydrated in a graded series of ethanol-buffer, then infiltrated and embedded in Spurr's resin according to the manufacturer's instructions (Electron Microscopy Sciences, Hatfield, PA). Thin sections were prepared using an LKB Huxley ultramicrotome (LKB-Produkter AB, Bromma, Sweden), mounted on uncoated 200-mesh copper grids, stained with 1% uranyl acetate followed by secondary staining with 0.1% lead citrate, and viewed with a Morgagni 268 electron microscope (FEI Company, Hillsboro, OR). Size calculations were carried out with AMTV542 Image Capture Engine software (Advanced Microscopy Techniques Corp., Danvers, MA).

Determination of the sensitivity of HoCV-1 amplification by RT-PCR

Plasmid DNA containing the full-length HoCV-1 cDNA cloned into the pCR®8/GW/TOPO® vector was recom-

bined with the pEXP3-DEST™ (Invitrogen, Carlsbad, CA) destination vector in the presence of LR Clonase™ (Invitrogen) using the LR recombination protocol supplied by the manufacturer. The reaction product was then used to transform One Shot® MAX Efficiency™ DH5α chemically competent *E. coli* (Invitrogen). Recombinants demonstrating both ampicillin-resistance and chloramphenicol-sensitivity were selected and grown overnight at 37 °C and 150 rpm in LB broth supplemented with 100 μg/mL ampicillin. Plasmid DNA was extracted using the QIAprep® Spin Miniprep kit (Qiagen, Valencia, CA) according to manufacturer's instructions. A 1-μg aliquot was transcribed using the AmpliScribe™ T7 High Yield Transcription kit (EPICENTRE® Biotechnologies, Madison, WI), yielding ~50 ng of RNA. RT-PCRs were performed with the QIAGEN® OneStep RT-PCR kit (Qiagen) using a range of serial dilutions (50 ng–500 ag) incorporated into the reaction either alone or as a complex mixture. Composite mixtures were prepared by combining the appropriate dilution with 400 ng total RNA extracted from an uninfected GWSS. Template RNA (1 μL) was reverse transcribed for each sample reaction by combining 5 μL QIAGEN OneStep RT-PCR Buffer, 1 μL dNTP mix (containing 10 mM of each dNTP), 5 μL 5× Q-Solution, 0.5 μL each forward and reverse primer (10 mM), 1 μL QIAGEN OneStep RT-PCR Enzyme Mix, and 1 μL RNasin® Ribonuclease Inhibitor (40 U/μL) (Promega) for a final reaction volume of 25 μL. The primers employed were designated HoCV-1F (5′-TCC GAG TTC TCA GCC AAA CT-3′) and HoCV-1R (5′-CGG CAT ATC GAA ATG AGG TT-3′), corresponding to nucleotides 8695–8714 and 9119–9138 located within the capsid (VP3) and 3′ untranslated region of the genome (Accession No. NC_008029). This mixture was incubated at 50 °C for 30 min, after which an initial PCR activation step was performed at 95 °C for 15 min to activate the HotStarTaq® DNA polymerase. Amplification was performed using 3-step cycling (35×) at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1 min followed by a final extension at 72 °C for 10 min. An aliquot of the resulting amplicons (20 μL each) was loaded onto a 1% agarose gel stained with EtBr and electrophoresed at 105V for 35 min. A gel image was acquired using a Kodak Image Station 440CF (Eastman Kodak, Rochester, NY). Samples were considered positive if a DNA band of the predicted molecular weight was visible. Detection limits for both the stand-alone and complex mixture assays were estimated using the "weight"-to-"mole" conversion calculator at http://molbiol.ru/eng/scripts/01_07.html.

Sample preparation and RT-PCR analysis

Upon receipt, insect tissue was removed from RNA*later*®, washed with 1X phosphate-buffered saline (PBS) (4 °C), and ground directly in Buffer RLT (Qiagen) using a sterile pestle. Total RNA extraction was performed using the RNeasy® Mini kit (Qiagen) according to the manufacturer's instructions. An equal concentration (~500 ng) of total RNA was retrotranscribed for each sample reaction and the resultant cDNA amplified using the OneStep RT-PCR kit (Invitrogen) in conjunction with the aforementioned HoCV-1 primer pair and thermal profile. Samples were considered positive when a visible amplicon (443 nucleotides) was present after electrophoretic separation.

Results and discussion

Analysis of HoCV-1 morphology via electron microscopy

Examination of transmission electron microscopy (TEM) images of sections obtained from the midgut region of *H. vitripennis* adults confirmed the presence of picorna-like virus particles presumed to be HoCV-1 (Fig. 1). Isometric particles averaging ~23 nm in diameter were observed throughout the cell, extending into the microvilli. While the mean particle size was slightly smaller than what is typically reported for members of the genus *Cripavirus*, the majority of the capsids measured fell within the appropriate range (27–30 nm). Given that the estimated diameters were not uniform, it is

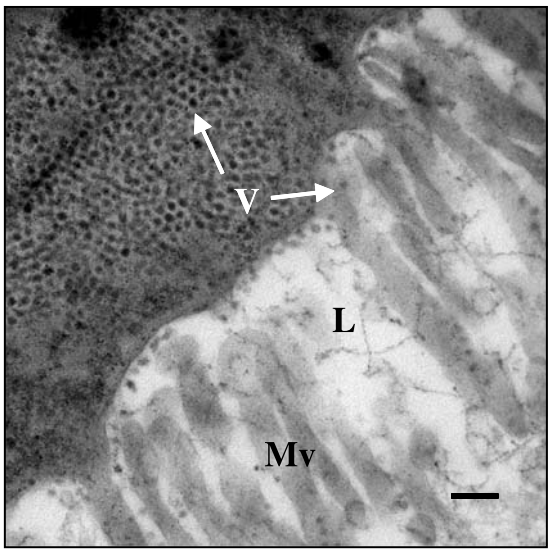

Fig. 1. Electron micrograph of midgut tissue dissected from an adult GWSS containing virus particles believed to be HoCV-1. Positive infection of the prepared tissue was confirmed by RT-PCR in parallel using primers specific for the capsid region of HoCV-1. *L* Gut lumen; *Mv* microvilli; *V* virus. *Scale bar* represents 100 nm

possible that the observed particles do not represent a single virus species (e.g., HoCV-1) but rather are an artifact of simultaneous infection of the insect with multiple viruses. No evidence of viral infection was noted in any of the other tissues sampled.

Sensitivity of the HoCV-1 RT-PCR assay

Unique diagnostic primers were designed against the capsid-coding region and a basic protocol developed for the detection of HoCV-1. To determine the sensitivity of the RT-PCR, a cDNA encoding the full length of the viral genome was retrotranscribed and an aliquot of the resultant RNA serial-diluted using 10-fold increments. Each dilution was incorporated into a reverse transcription reaction as a stand-alone template or as a complex mixture and then subjected to amplification by way of PCR. Figure 2 shows the detection sensitivity of RT-PCR on these samples, with a threshold of 500 ag or ~95 genome equivalents (geq). These results are consistent with other reports examining the lower limits of similar RT-PCR assays. For example, sensitivities with acute bee paralysis virus (*ABPV*) and black queen-cell virus (*BQCV*) were on the order of 1600 and 130 geq, respectively [1]. The 10-fold

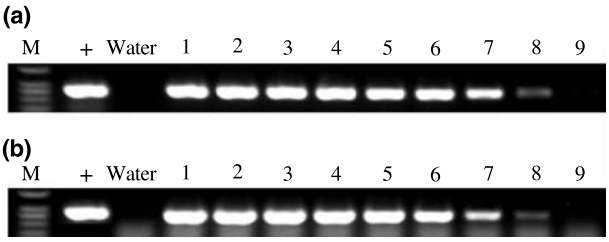

Fig. 2. Detection sensitivity of HoCV-1 by RT-PCR. RNA stock (50 ng/μL) obtained through transcription of a cDNA encoding the full genome of HoCV-1 was serially diluted (10-fold/dilution) to produce eight working solutions with concentrations ranging from 5 ng/μL to 500 ag/μL. One microliter of each working solution was used in a one-step RT-PCR reaction either alone (**a**) or as a complex mixture (**b**) (i.e., HoCV-1 genomic RNA at the specified dilution pooled with 400 ng total RNA extracted from an uninfected GWSS). *M*, DirectLoad™ marker (Sigma-Aldrich, St. Louis, MO); + *H. vitripennis* collected from *Citrus sinensis* in Riverside, California; *Water* water/no template control; *1* 50 ng/μL dilution; *2–9* 10^{-1}–10^{-7} HoCV-1 dilution in decreasing concentration

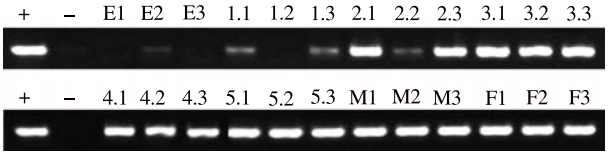

Fig. 3. Survey of individual growth stages of *H. vitripennis* for the presence of HoCV-1. + *H. vitripennis* collected from *Citrus sinensis* in Riverside, California; – water/no template control; *E* egg; *1–5* 1st through 5th instar; *M* male adult; *F* female adult. Three biological replicates were processed for each sex/lifestage. An equal concentration (500 ng) was incorporated into each one-step RT-PCR reaction and a uniform volume (18 μL) loaded onto a 1% agarose gel

variation noted between the two assays was attributed to the difference in the size of the amplified fragments, with the shorter fragment yielding a positive result with decreased titers. In addition to amplimer length, cycling conditions and enzyme selection can also influence detection sensitivity. For example, in the current study, the one-step method was found to be superior to standard RT-PCR not only with respect to reaction setup time, but also in degree of sensitivity (data not shown).

Sex and lifestage susceptibility of GWSS to HoCV-1 infection

A product corresponding to HoCV-1 was noted in both sexes and all developmental stages of GWSS, including eggs (Fig. 3). Variation in band intensities observed between the lifestages (e.g., egg versus 3rd-instar) suggests that differences in viral load may exist. Although no mode(s) of transmission could be conclusively accepted/rejected, these results suggest that infection may be maintained through both transovarial and transstadial transmission.

Survey of the prevalence of HoCV-1 in Florida GWSS

Specifics regarding collection date, locality and number of individuals tested at each site as well as the data obtained from RT-PCR detection of HoCV-1 are presented in Table 1. Viruliferous samples were found only in Gadsden and Suwannee counties, two of the three localities in which GWSS

Table 1. Survey of *Homalodisca vitripennis* populations for the presence of HoCV-1

Date	Locality (County, State)	Host plant(s)	N[a]	N(+)[b]
28-Aug-2004	Grady Co., GA	*Ilex* spp., *Lagerstroemia indica*	35	35
14-Jul-2005	Honolulu Co., HI	*Spathodea companulata*	11	0
03-Jun-2006	Gadsden Co., FL	*L. indica*	30	4
04-Jun-2006	Jefferson Co., FL	*L. indica*	30	0
04-Jun-2006	Suwannee Co., FL	*L. indica*	30	3
26-Jun-2006/01-Jul-2006	Okeechobee Co., FL	*L. indica*	11	0
03-Jun-2006	Marion Co., FL	*L. indica*	2	0
02-Jul-2006	Citrus Co., FL	*L. indica*	2	0
06-Jul-2006/19-Jul-2006	Indian River Co., FL	*C. sinensis*	11	0
08-Jul-2006	St. Lucie Co., FL	*L. indica*	11	0
23-Jul-2006	Clay Co., FL	*L. indica*	14	0
23-Jul-2006	Duval Co., FL	*L. indica*	5	0
23-Jul-2006	Putnam Co., FL	*L. indica*	2	0
23-Jul-2006	Nassau Co., FL	*L. indica*	1	0
30-Jul-2006	DeSoto Co., FL	*L. indica*, *Magnolia virginiana* 'Sweetbay', *Ulmus parvifolia*	16	0
05-Aug-2006	Robeson Co., NC	*L. indica*	13	1
07-Aug-2006	Dare Co., NC	*Eleagnus pungens* 'Ebbingei'	7	1
10-Aug-2006	Georgetown Co., SC	*L. indica*	10	6

[a] Denotes number of individuals tested.
[b] Denotes number of individuals that tested positive for HoCV-1.

were most abundant. The later personal account is congruent with a distribution study conducted by Hoddle et al. [6] which found that significantly more GWSS inhabit north Florida than central and south Florida. This reduction in host population density may correspond to the apparent absence of HoCV-1 in the central and south Florida counties surveyed. Alternatively, these two populations (north vs. central/south Florida) could have evolved from disparate lineages and continue to exist as isolated entities which do not interact, thus being segregated not only geographically but genetically as well. A large-scale study incorporating molecular techniques such as inter-simple sequence repeat (ISSR) and mitochondrial DNA haplotype analysis is currently underway to examine the population genetics of *H. vitripennis* from several regions throughout the state (methods reviewed in [4, 16]).

Incidence of HoCV-1 infection in GWSS with geographically disparate origins

Infected GWSS were found in Georgia, South Carolina, and North Carolina with the incidence of infection ranging from 8 to 100% (Table 1). Conversely, our assay failed to detect HoCV-1 in any of the GWSS collected from the island of Oahu. Virus infection was distributed among populations regardless of the host plant from which the insect was harvested. The lack of infected individuals in Hawaii can be ascribed to several different variables. For example, there may be certain climatic constraints precluding virus replication in the tropics that are not a factor in more temperate regions. Also, paralleling the south Florida population, the *H. vitripennis* population found in Oahu may have originated from an alternate lineage than those in north Florida, Georgia and the Carolinas and was either introduced as a "healthy" population or possesses a resistance gene/genetic element which confers immunity to HoCV-1 infection.

Occurrence of HoCV-1 in alternate sharpshooter vector species

Adults of two additional sharpshooter vector species, *H. insolita* and *O. nigricans*, collected in north

Table 2. Survey of alternate leafhopper vector species for the presence of HoCV-1

Tissue source (Genus species)	Date	Locality (County, State)	Host plant(s)	N[a]	RT-PCR results
D. minerva	10-Jun-2005	Mendocino Co., CA	Mixed riparian habitat (primarily *Vitis californica*)	12	−
	10-Jun-2005	Sonoma Co., CA	Mixed riparian habitat (primarily *Artemisia douglasiana*)	23	−
G. atropunctata	10/13-Jun-2005	Alameda Co., CA	*Ocimum basilicum*, *Rosa* spp.	27	−
	10-Jun-2005	Napa Co., CA	Mixed riparian habitat (primarily *A. douglasiana* and *V. californica*)	12	−
	10-Jun-2005	Sonoma Co., CA	Mixed riparian habitat (primarily *A. douglasiana* and *V. californica*)	47	−
H. insolita	04-Mar-2004	Gadsden Co., FL	*Sorghum halepense*	10	+
	02-Sep-2005	St. Lucie Co., FL	*S. halepense*	6	−
O. nigricans	22-Mar-2004	Gadsden Co., FL	*Ilex* × *meserveae* 'China Girl'	9	+
	28-Aug-2004	Grady Co., GA	*Ilex* spp.	12	+

[a] Denotes number of individuals assayed as a pooled population.

Florida were also demonstrated to be natural hosts for HoCV-1 (Table 2). However, neither *D. minerva* nor *G. atropunctata* tested positive for the virus. These findings suggest that while HoCV-1 is not limited to *H. vitripennis*, infection is not ubiquitous to all sharpshooter genera and may even be restricted to members of the tribe Proconiini.

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